Transgenic Complementation of Leptin-Receptor Deficiency

I. Rescue of the Obesity/Diabetes Phenotype of LEPR-Null Mice Expressing a LEPR-B Transgene

Timothy J. Kowalski, Shun-Mei Liu, Rudolph L. Leibel, and Streamson C. Chua, Jr.

Mice homozygous for the *Lepr^{db3J}* (*db^{3J}*) mutation are null for all known isoforms of the leptin receptor (LEPR). These animals are obese, hyperphagic, cold intolerant, insulin resistant, and infertile. Mice homozygous for the *Lepr^{db}* (*db*) mutation (lacking the B isoform only) have the same phenotype as *db^{3J}* animals. To better understand the function(s) of the LEPR isoforms in vivo, we generated *db^{3J}/db^{3J}* and *db/db* mice bearing a transgene (neuron-specific enolase [NSE]-Rb) expressing the B isoform of LEPR, the isoform capable of activating the signal transducer and activator of transcription (STAT) pathway, under the control of the neuron-specific enolase enhancer/promoter. The NSE-Rb transgene was expressed in the brain, with low levels of expression in adrenals, testis, and white adipose tissue. LEPR-B transgene expression in NSE-Rb *db^{3J}/db^{3J}* mice partially corrected the increased fat mass, hyperphagia, and glucose intolerance while restoring fertility in males and rescuing the cold intolerance in both sexes. The body weights of NSE-Rb transgenic mice that possessed the full complement of short LEPR isoforms (NSE-Rb *db/db* mice) were similar to those of NSE-Rb *db^{3J}/db^{3J}* mice, suggesting that the short LEPR isoforms play little role in body weight regulation. Based on quantitative analysis of hypothalamic neuropeptide gene expression in the transgenic animals, we infer full restoration of leptin sensitivity to proopiomelanocortin (POMC) neurons, partial correction of leptin sensitivity in agouti gene–related protein (AGRP)/neuropeptide Y (NPY) neurons, and a lack of effect on leptin sensitivity of melanin concentrating hormone neurons. Thus, hypothalamic POMC and AGRP/NPY neurons are primary candidates as the mediators of the effects of the NSE-Rb transgene on energy homeostasis, ingestive behavior, the neuroendocrine system, and glucose metabolism. *Diabetes* 50:425–435, 2001

The leptin-leptin receptor axis is an important regulator of feeding behavior, energy expenditure, body composition, and glucose homeostasis. Deficiencies of leptin (*Lep$^{ob/ob}$* mice) or the leptin receptor (*Lepr$^{db/db}$* mice, *Lepr$^{fa/fa}$* rats) produce obesity in both humans and rodents due to hyperphagia, decreased energy expenditure, and preferential partitioning of calories into adipose tissue triglycerides (1). Leptin is expressed in and secreted predominantly by adipocytes (2), and it circulates at a concentration that is proportional to fat mass in both humans and rodents (3). Changes in fat mass produce changes in plasma leptin concentrations, suggesting that circulating leptin provides a humoral signal of the mass of adipocyte energy stores (3). Leptin may serve primarily to mediate a response to caloric deprivation and attendant loss of fat mass (4). A starvation response is evidenced by acute downregulation of leptin expression after food deprivation and upregulation with refeeding (5). Consistent with this role, repetitive administration of leptin to hypoleptinemic, starved mice attenuates many of the neuroendocrine changes associated with starvation (6).

Animals with a null mutation in *Lep* (*Lep$^{ob/ob}$* mice) are obese, hyperphagic, cold intolerant, insulin resistant (and frequently glucose intolerant), have an abnormally regulated hypothalamic-pituitary-adrenal axis, and are infertile (1). Peripheral administration of recombinant leptin to *ob/ob* mice completely reverses the obesity by decreasing food intake and increasing energy expenditure (7). Additionally, peripheral leptin treatment in *ob/ob* mice restores glucose tolerance, cold tolerance, and fertility (7,8). The effects of leptin on feeding behavior, energy balance, and glucose homeostasis appear to be centrally mediated, as these actions of peripherally administered leptin can be produced with intracerebroventricular injections at doses too low to affect circulating leptin concentrations (1–3 μg) (9). Although central leptin administration appears sufficient to reverse the obesity/diabetes phenotype in *ob/ob* mice, several groups have reported effects of leptin in isolated peripheral tissues in vitro (e.g., pancreas [10], muscle [11], liver [12], and adipose tissue [13]). The relevance of these actions of leptin outside

From the Department of Pediatrics, Division of Molecular Genetics and Naomi Berrie Diabetes Center, Columbia University College of Physicians and Surgeons, New York, New York.

Address correspondence and reprint requests to Streamson C. Chua, Jr., MD, PhD, Department of Pediatrics, Division of Molecular Genetics, Columbia University College of Physicians and Surgeons, Russ Berrie Medical Science Pavilion, 1150 St. Nicholas Ave., New York, NY 10032. E-mail: sc569@columbia.edu.

Received for publication 20 July 2000 and accepted in revised form 11 October 2000.

Current address for T.J.K.: Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033.

AGRP, agouti gene–related protein; CNS, central nervous system; JAK, Janus kinase; LEPR, leptin receptor protein; MCH, melanin concentrating hormone; NAL, naso-anal length; NPY, neuropeptide Y; NSE, neuron-specific enolase; PCR, polymerase chain reaction; POMC, proopiomelanocortin; RT, reverse transcription; STAT, signal transducer and activator of transcription; SV40, simian virus 40.

of the nervous system to the normal in vivo physiology of energy and glucose homeostasis are currently unclear.

The leptin receptor protein (LEPR) is a member of the class I cytokine receptor superfamily that activates the JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathway (14,15). Multiple splice variants of *Lepr* mRNA have been identified, each encoding an identical ligand binding domain, but differing by virtue of the presence or absence of a transmembrane domain or a complete cytosolic domain (14,16). Of the four "short" splice variants (LEPR-A, C, D, and E) identified, the LEPR-A isoform is the quantitatively dominant molecular species and is expressed in all tissues examined (17). All of the LEPR isoforms that possess a cytosolic domain (A, B, C, and D) also contain the same JAK binding motif encoded in coding exon 17. However, only the LEPR-B isoform contains a Box 3 motif for STAT activation in its terminal cytosolic domain (14,16,18). The LEPR-B isoform is expressed ubiquitously, but at levels much lower than the A isoform. An exception to this is the hypothalamus, where LEPR-B constitutes up to 35% of *Lepr* transcripts (17,19).

Consistent with its homology to the cytokine receptor family, LEPR-B is capable of dimerizing and activating STAT3 and STAT5 proteins in heterologous systems (19,20) Thus, the LEPR-B isoform is proposed to be the physiologically relevant signaling isoform in the regulation of energy balance. Consistent with this inference, *db/db* mice, which lack only functional LEPR-B, develop an obesity/diabetes syndrome apparently identical to *ob/ob* mice and are unresponsive to peripheral or central leptin administration (9). Furthermore, mice and rats possessing allelic mutations in the *Lepr* gene that affect all LEPR isoforms (db^{3J} [21], db^{Pas} [22], db^{NCSU} [23], *fa* [24], and *fa-f* [25]) exhibit the *db/db* obesity/diabetes phenotype. Studies in vitro have revealed LEPR-A signaling activities (e.g., immediate-early genes [26]) but the physiological relevance of this pathway to in vivo energy and glucose homeostasis is not clear.

As an initial step toward analyzing the function(s) of leptin receptor isoforms in vivo, we have created mice bearing a transgene expressing the B isoform of the leptin receptor under the control of the neuron-specific enolase (NSE) promoter (27). We find that the expression of this transgene (NSE-Rb) in db^{3J}/db^{3J} mice, otherwise null for all leptin receptor isoforms, partially rescues the obesity/diabetes and infertility phenotypes, while correcting the cold intolerance of these mutants. Quantitative analysis of the neuropeptides expressed by three hypothalamic neuronal populations indicates that the NSE-Rb transgene affects the expression of proopiomelanocortin (POMC), neuropeptide Y (NPY), and agouti gene-related protein (AGRP), whereas expression of melanin concentrating hormone (MCH) is unaffected by the transgene. We postulate that the partial correction of the obese/diabetes phenotype is due to the restoration of leptin sensitivity to POMC and AGRP/NPY hypothalamic neurons. We also postulate that the residual obesity and insulin resistance of NSE-Rb db^{3J}/db^{3J} mice may be due to the persistent abnormal activity of hypothalamic NPY/AGRP and MCH neurons. Furthermore, expression of NSE-Rb in animals lacking only LEPR-B (*db/db* mice) results in body weights similar to those seen in NSE-Rb db^{3J}/db^{3J} mice, suggesting that short LEPR isoforms play a limited role in body weight regulation. These experiments indicate the feasibility of using molecular genetic tools to dissect the roles of specific neuronal populations in energy balance, body weight regulation, and appetitive behavior.

RESEARCH DESIGN AND METHODS

Transgene construction. The full-length cDNA encoding mouse LEPR-B was obtained from D. White (Millennium Pharmaceuticals) in the pMET7 expression vector (14). The SR-α promoter of the pMET7 vector was excised and replaced with a synthetic polylinker containing restriction sites for *Pac*I, *Pme*I, and *Asc*I. A 1.8 kb *Sac*I/*Xho*I fragment containing the NSE (*Eno2*) promoter/enhancer (27) (gift from Dr. G. Sutcliffe) was inserted into the *Pac*I site. A rat growth hormone intron (generated by polymerase chain reaction [PCR]) was inserted into the *Asc*I site between the NSE promoter/enhancer and the LEPR-B cDNA (Fig. 1) to enhance transcription of the LEPR-B transgene (28,29). A simian virus 40 (SV40) late polyadenylation signal, present in the pMET7 vector, was 3′ of the LEPR-B cDNA. The transgene construct was excised using *Hind*III and *Sac*I and purified from the vector by velocity sedimentation in a linear 5–25% sodium chloride gradient. Fractions containing the construct were pooled and dialyzed against TE (1 mmol/l Tris, pH 8.0, 0.1 mmol/l EDTA).

Production of transgenic mice. DNA was microinjected into fertilized C57BL/6J×CBA F2 oocytes. Surviving oocytes were transferred to the oviducts of pseudopregnant foster mothers according to previously described methods (30). These procedures were performed by the Herbert Irving Comprehensive Cancer Center Transgenic Mouse Facility at Columbia University. Sixteen progeny were obtained, two of which were positive for the transgene by PCR analysis of ear-clip DNA (see below; male #3 and male #12). The two male founders were mated with C57BL/6J.129-A^{wJ} db^{3J} congenic females (see below). Both males transmitted the transgene to their progeny. The expression of the transgene was evaluated in progeny by reverse transcription (RT)-PCR analysis using two primer pairs: the 5′ end was amplified using primers based on sequences after the NSE transcription start site and a sequence specific to LEPR-B, and the 3′ end was amplified using primers based upon the SV40 polyA sequence and a LEPR-B–specific sequence. The progeny of male #3 expressed full-length LEPR-B mRNA. The progeny of male #12, however, expressed a truncated LEPR-B mRNA consisting only of the 3′ end. Thus, only line #3 was used for further analysis and line #12 was discontinued.

Line #3 progeny were backcrossed to C57BL/6J.129-A^{wJ}/A^{wJ} db^{3J}/+ congenics to generate N3E and N4E (N3 and N4 equivalent, respectively) experimental animals. The B6 congenic line used (N10F4 or N10F5 C57BL/6J.129-$A^{wJ}db^{3J}$ mice) carrying the db^{3J} mutation was developed by this laboratory from 129/J-db^{3J} animals provided by Dr. Ed Leiter (Jackson Labs). The db^{3J} mutation was crossed onto white-bellied agouti C57BL/6J-A^{wJ} mice (co-isogenic with C57BL/6J) to enable easy visual discrimination from black C57BL/6J mice harboring the *db* mutation (see below). To generate NSE-Rb *db/db* animals, transgenic progeny of initial matings (line #3) were initially crossed with FVB.C57BLKS/J-*db* congenic females (congenic FVB.BLKS-*db* animals were generated by this laboratory and were used in initial matings to increase the number of transgenic progeny). Progeny from this mating were backcrossed to C57BL/6J-*m db* congenic mice to generate N1 and N2 experimental animals. The B6 congenic line used (N10F4 or N10F5 C57BL/6J-*m db* mice) carrying the *db* mutation was developed by this lab using N8 C57BL/6J-*m db*/+ + breeders obtained from Jackson Labs. This is the stock in which the misty and diabetes mutations are maintained in the same phase. However, as our breeding progressed, we have observed numerous examples of recombination between misty and diabetes, i.e., obese animals that were not misty. Therefore, we have not specified the genotype at the misty locus for the experimental animals.

All animals used were maintained under barrier condition at 22°C with a 12h:12h light-dark cycle (0700–1900). Animals had free access to rodent food (Picolab Mouse Diet 20: 55% carbohydrate, 20% protein, 9% fat; PMI Nutrition International) and water unless specified. Pathogen testing is done on a quarterly basis, and all serologies have been negative to date.

Preparation and analysis of genomic DNA. Transgenic mice were identified using PCR analysis of genomic DNA isolated from ear clips or tail clips. Tissue was digested in a buffer of 1% SDS, 1 mmol/l EDTA, 10 mmol/l Tris, pH 8.0 with 100 μg/ml proteinase K at 65°C overnight, followed by phenol/chloroform extraction and DNA precipitation. Amplification with primers NSE-Ex1B (5′-CCACCGGCTGAGTCTGCAGT-3′) and rGHINT-R (5′-TTGGCGCGCCGAGAGT CTAGAAAGAGAGACA-3′) produced a 242-bp fragment if the transgene is present and no product if the transgene is absent. The genotype at *Lepr* was determined by PCR analysis of ear-clip DNA as follows: the 17-bp deletion of the $Lepr^{db3J}$ mutation was identified using the following primers:

Ex-11R: 5′-GTTCTTCAGTCACGCTTGA-3′
Lepr-53: 5′-CATGAGGTATTCGATGCAAAG-3′

The *Lepr*^{db} mutation was identified by an *Rsa*I restriction site introduced by the mutagenic primer db-F1 and db-R (31):

db-F1: 5′-AGAACGGACACTCTTTGAAGTCTC-3′
db-R: 5′-CATTCAAACCATAGT-TTAGGTTTGTGT-3′

Semiquantitative RT-PCR for LEPR and neuropeptide transcripts. Total RNA was isolated from tissues extracted with guanidinium salts (32). Two micrograms of total RNA was reverse transcribed using MMLV Reverse Transcriptase (Life Technologies) and an anchored oligo-dT primer adaptor (Not-PA, Promega) as described by the manufacturer. Expression of the NSE-Rb transgene was evaluated using a nested PCR strategy. Two microliters of cDNA (20 ng equivalents of total RNA) was amplified with primers mObr-25 (5′-GATTTCACCACAACTTTCATTCTC-3′) and Not-PA using the following conditions: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for 20 cycles. One microliter of this PCR reaction was amplified with primers Lepr-Rb1 (5′-CCTGTTCCACG CACAGTCAC-3′) and Not-PA using the same PCR conditions as above for 35 cycles. A 255-bp fragment was generated if the NSE-Rb transcript was present. This protocol, however, did not amplify the endogenous LEPR-B transcript. The integrity of cDNA was examined by PCR amplification using a primer complementary to actin (5′-CTGGAGAAGAGCTATGAGCTGCCT-3′) and Not-PA, generating a 510-bp fragment (β-actin) and a 691-bp fragment (α_{sk}-actin).

One to two micrograms of hypothalamic RNA was reverse transcribed with Superscript II (Life Technologies) with 200 ng of the Not primer-adaptor and 0.5 mmol/l dNTPs in a total volume of 20 µl. After incubation at 45°C for 60 min, the samples were diluted to 200 µl with sterile water and incubated at 65°C for 20 min to inactivate the reverse transcriptase. All samples being compared were reverse transcribed simultaneously to minimize differences in the efficiency of cDNA synthesis. To minimize variation due to sample handling, all samples to be compared were amplified on the same thermal cycler, and all amplification products for a given pair of primers were electrophoresed on the same gel. Amplification of LEPR-B cDNA was performed with isoform-specific primers for 40 cycles (94°, 55°, and 72° with 30 s at each temperature):

mObr-G: CCC ATC GAG AAA TAT CAG
mLepr-60: GGC TCC AGA AGA AGA GGA CC

Amplification of actin cDNA was done for 26 or 28 cycles (same cycling parameters as for LEPR-B cDNA) with the following primers:

Actin-1: CTG GAG AAG AGC TAT GAG CTG CCT
Actin-3: CTC CTG CTT GCT GAT CCA CAT CTG

Amplification of neuropeptide cDNAs was done with the following primers:

AGRP	AGRP-1: AGG GCA TCA GAA GGC CTG ACC AGG
	AGRP-2: CTT GAA GAA GCG GCA GTA GCA CGT
MCH	MCH-1: GAG TCC ACA CAG GAA AAG AG
	MCH-2: CAG CAG GTA TCA GAC TTG CC
NPY	NPY-61: ATG CTA GGT AAC AAG CGA ATG GGG
	NPY-CPON: TGA AAT CAG TGT CTC AGG GCT GGA
POMC	POMC-1: GTG CCT GGA GAG CAG CCA GT
	POMC-2: GAA GTT CCT CGG GGA CAG TC

The cycling parameters for three neuropeptide cDNAs (AGRP-32 cycles, NPY-30 cycles, and POMC-32 cycles) were 94° – 60° – 72°C for 30 s at each temperature plateau, whereas MCH cDNA was amplified for 26 cycles with an annealing temperature of 55°C. The optimal cycle number was determined from the *db*^{3J}/+ cDNA pool to place the linear range of amplification for 20-fold dilutions of the cDNA samples. All of the primer pairs are designed to span at least one intron to distinguish between amplification products derived from genomic DNA and cDNA. All primer pairs were empirically shown not to amplify any products from genomic DNA using the specified conditions. Primers were based on mouse and rat cDNA sequences: β-actin (33), AGRP (34), MCH (33), NPY (GenBank Accession # AF273768), and POMC (35). The rat MCH sequence was aligned to two mouse MCH-like expressed sequence tags for optimal homology to mouse MCH mRNA.

For quantitative analysis of LEPR-B transcripts, serial twofold dilutions (6 for *db*^{3J}/+ and *db*^{3J}/*db*^{3J} and 12 dilutions for NSE-Rb *db*^{3J}/*db*^{3J}) were amplified in duplicate with LEPR-B and actin primers. For quantitation of neuropeptide cDNAs, the samples were diluted 1:20 with water, and 5 µl of the diluted samples was used for amplification in duplicate in 25 µl reactions. A standard curve was generated from serial twofold dilutions (8–10 dilutions in duplicate) of a pool of the cDNA samples from *db*^{3J}/+ animals. The procedure has been previously described (23). The amplified products were size fractionated on an agarose gel (2% agarose + 1% low-melting-temperature agarose) containing ethidium bromide. DNA-bound fluorescence was quantified with an 8-bit video camera using QuantOne software (BioRad). The amount of amplified product is determined by the following equation:

$$T_j = T_0 * (1 + E_t)^j \quad (1)$$

where T_0 is the initial amount of template, j is the number of PCR cycles, E_t is the mean efficiency of amplification per cycle, and T_j is the amount of product after j cycles (36). Taking the logarithm of Eq. 1, we obtain:

$$\log T_j = \log T_0 + j \log (1 + E_t) \quad (2)$$

Because Eq. 2 applies within the linear range of amplification, a plot of log T_0 to log T_j should yield a straight line. Samples with equivalent amounts of the template mRNA will yield superimposable lines with similar slopes and intercepts. Samples with varying amounts of the template mRNA will yield parallel lines with identical slopes (identical amplification efficiencies) but different intercepts (varying T_0). Although this procedure does not provide absolute quantification, relative differences in the fractional contents of a given mRNA species between various samples can be inferred by calculations that solve for T_0 at equivalent T_j for different samples. The inclusion of a standard housekeeping gene as an internal standard provides data equivalent to high-throughput real-time RT-PCR methods (37,38).

Measurement of blood glucose, leptin, and insulin. Tail blood for glucose, insulin, and leptin measurements was collected from fed and/or 24-h fasted animals between 0800 and 1200 while they were lightly restrained. Glucose was measured using the glucose-oxidase method (Glucometer Elite; Bayer, Elkhart, IN). Serum insulin was quantified using a commercial radioimmunoassay kit with rat insulin as standard (Linco, St. Louis, MO). Serum leptin was quantified using a commercial enzyme-linked immunosorbent assay kit (Crystal Chem, Chicago).

Measurement of fat pad weights and BMI. Animals were killed by CO_2 asphyxiation and bled by cardiac puncture for measurement of leptin and insulin. The naso-anal length (NAL) was measured and the BMI calculated as the body weight/NAL^2 (g/cm^2). The right inguinal, retroperitoneal, and perigonadal depots and the mesenteric depot were dissected and weighed. The total adipose depot weight of each animal was defined as the sum of ([right inguinal, retroperitoneal, and perigonadal depots] × 2) + (mesenteric depot).

Glucose tolerance test. Tail blood was collected from 15- to 20-week-old mice before and after 24 h of food deprivation for measurement of whole blood glucose concentrations. After 24-h food deprivation, glucose (1 mg/g$^{0.75}$) was administered intraperitoneally, and tail blood (5 µl) was collected for glucose measurements (while mice were lightly restrained) before and 20, 40, 60, and 90 min after glucose administration. Doses were calculated to a power function of body weight to accurately reflect metabolic mass.

Cold tolerance test. Core (rectal) temperature of individually housed mice was measured using a thermocouple probe (IT-18, Physitemp) connected to a thermocouple thermometer (BAT-10, Physitemp) at 22°C ambient and at 0.5, 1, 1.5, and 2 h after being placed at 4°C ambient. Mice were manually restrained during probe insertion.

Measure of food intake. Daily pelleted mouse chow (Picolab Rodent Chow 5058) intake of individually housed mice was calculated from 24-h, 48-h, or 72-h intake measures. Food intake was expressed as g chow/day and g chow · (g body wt)$^{-0.75}$ · day^{-1}.

Statistical analysis. All values are expressed as means ± SE, unless otherwise indicated. Statistical differences among genotypes were assessed by a Kruskal-Wallis H-test followed by a Mann-Whitney *U* test where appropriate. A paired Student's *t* test was used to assess statistical differences between fed and fasted states. A one-tailed test with a P value <0.05 was considered significant.

RESULTS

Transgene construction and generation of NSE-Rb transgenic mice. The NSE-Rb construct (Fig. 1) places the LEPR-B cDNA under the control of the NSE (*Eno2*) promoter (27). A rat growth hormone intron was placed between the promoter and the cDNA to enhance expression of the transgene. Of two transgenic lines produced by the oocyte injections, one line was found to contain the full NSE-Rb transgene. This line was used in all subsequent experiments. The second line was lacking the NSE promoter by PCR analysis of genomic DNA and was discontinued.

Nested RT-PCR analysis showed that, as expected, the transgene is expressed in all brain regions examined (hypo-

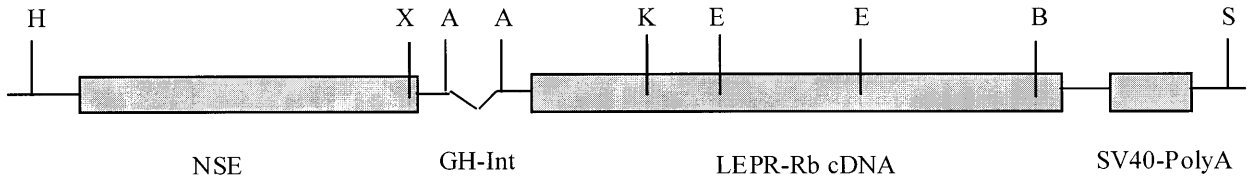

FIG. 1. Structure of the NSE-Rb transgene. NSE, neuron-specific enhancer/promoter; GH-Int, rat growth hormone intron; SV40-PolyA, SV40 polyadenylation signal; LEPR-B cDNA, full-length mouse cDNA for LEPR-B. Restriction sites: H, *Hind*III; X, *Xho*I; A, *Asc*I; K, *Kpn*I; E, *Eco*RI; B, *Bam*HI; S, *Sma*I.

thalamus, cortex, hindbrain, and cerebellum) (Fig. 2*A* and *B*). Low levels of NSE-Rb expression were seen in adrenals, white adipose tissue, and testis. Of note, there was no detectable LEPR-B mRNA from the transgene in the pancreas or in brown adipose tissue. Comparison of LEPR-B expression from the NSE-Rb transgene and the endogenous *Lepr* gene was performed by semiquantitative RT-PCR of hypothalamic RNA from mice of three genotypes: db^{3J}/+, db^{3J}/db^{3J}, and NSE-Rb db^{3J}/db^{3J}. Regression analysis was performed on the amplification products from serial twofold dilutions of hypothalamic cDNA with correction for actin content. The analysis indicated that the NSE-Rb transgene is being expressed at 14-fold (relative to db^{3J}/db^{3J}) to 30-fold (relative to db^{3J}/+) higher concentrations in the hypothalamus than is the endogenous *Lepr* gene (Fig. 2*C*). The large differences in transcript concentrations derived from the transgene and the endogenous gene account for the failure to amplify a fragment from the endogenous gene using the nested PCR approach that used an anchored oligo-dT downstream primer. We were unable to amplify any fragments that contained the 17-bp deletion of the db^{3J} mutation from db^{3J}/db^{3J} mice (data not shown), indicating that the amplified LEPR cDNA products from db^{3J}/db^{3J} tissues are not full-length LEPR transcripts.

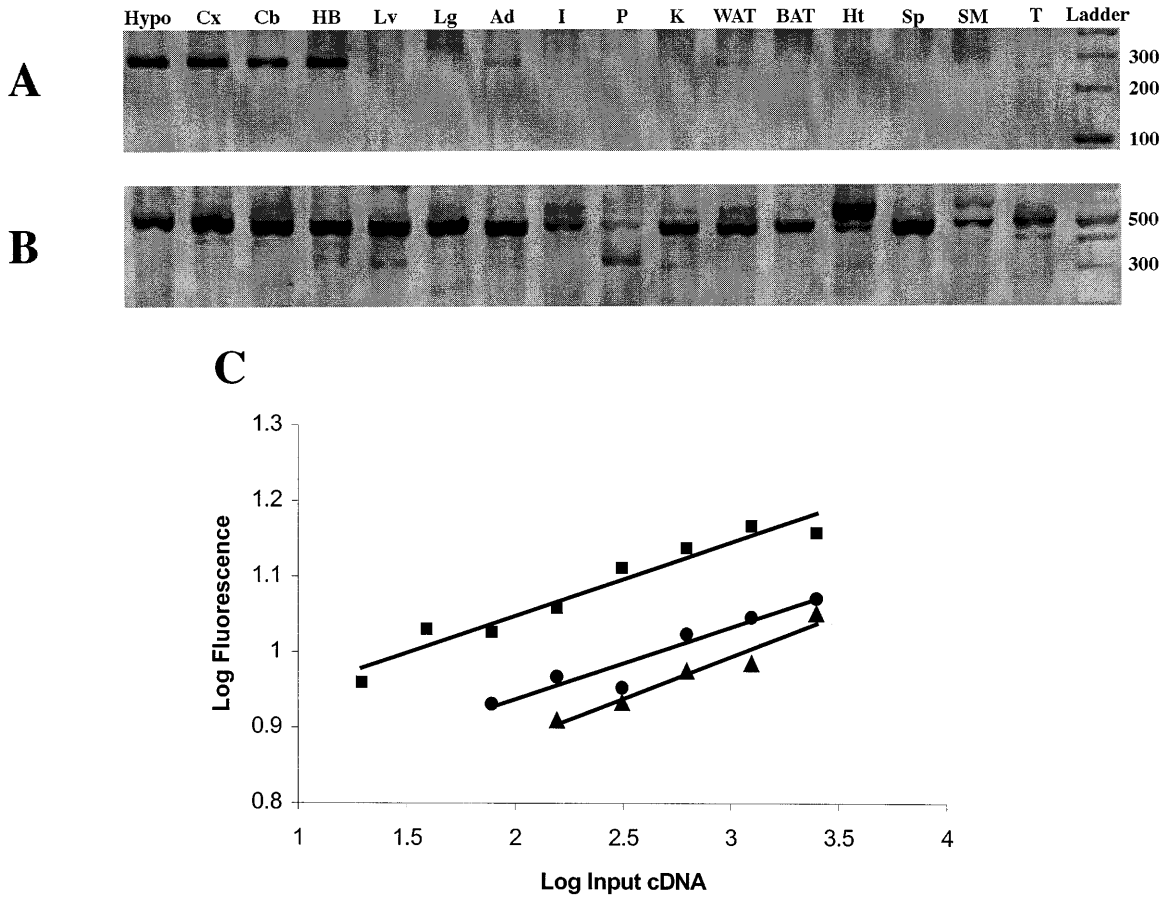

FIG. 2. *A*: NSE-Rb expression analysis in several tissues of an NSE-Rb db^{3J}/db^{3J} mouse. A 255-bp PCR fragment is generated from tissue cDNA if the NSE-Rb transgene is expressed. *B*: Assessment of actin expression as control. Fragments derived from β-actin transcripts (510 bp) or α_{sk}-actin (691 bp) were produced. Lanes correspond to the tissues in *A*. Hypo, hypothalamus; Cx, cortex; Cb, cerebellum; HB, hindbrain; Lv, liver; Lg, lung; Ad, adrenals; I, intestine; P, pancreas; K, kidney; WAT, white adipose tissue; BAT, brown adipose tissue; Ht, heart; Sp, spleen; SM, skeletal muscle; T, testis. *C:* Comparison of hypothalamic LEPR-B expression from the NSE-Rb transgene and the endogenous *Lepr* gene. Regression functions for NSE-Rb db^{3J}/db^{3J} (■) (0.0975 log [input RNA] + 7.1236), db^{3J}/db^{3J} (●) (0.0954 log [input RNA] + 5.577), and db^{3J}/+ (▲) (0.111 log [input RNA] + 0.659).

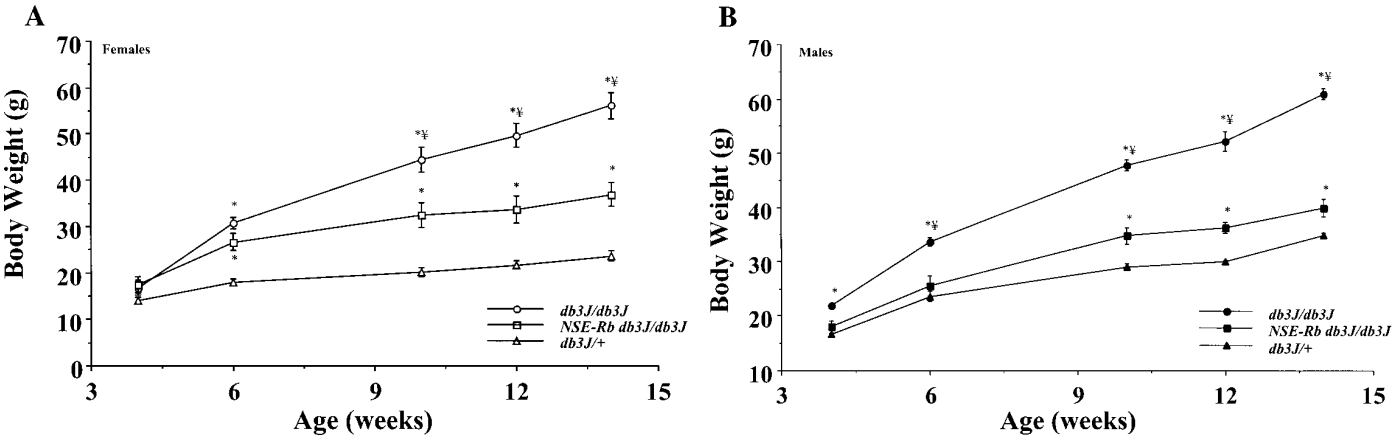

FIG. 3. *A*: Growth curves of female db^{3J}/+ (n = 6), db^{3J}/db^{3J} (n = 7), and NSE-Rb db^{3J}/db^{3J} (n = 5) mice. *B*: Growth curves of male db^{3J}/+ (n = 3), db^{3J}/db^{3J} (n = 3), and NSE-Rb db^{3J}/db^{3J} (n = 3) mice. *Significantly different from db^{3J}/+ mice. ¥Significantly different from NSE-Rb db^{3J}/db^{3J} mice.

The NSE-Rb transgene was backcrossed three times (N4 equivalents, ~93% C57BL/6J) to two *Lepr*^{db} congenic lines, C57BL/6J – A^{wJ}/db^{3J} and C57BL/6J – *m db*, to generate a more uniform genomic composition and to allow direct comparisons to published literature using the C57BL/6J strain. The db^{3J} mutation is maintained on the co-isogenic B6-A^{wJ} line to allow visual discrimination between the two congenic lines (the B6-*m db* line produces black mice whereas the B6-A^{wJ}/db^{3J} line produces white-bellied agouti mice). All experimental animals were typed for the presence/absence of the transgene, which was maintained in the hemizygous state. All matings were between a transgenic mouse and a nontransgenic mate.

The NSE-Rb transgene partially corrects the obesity and hyperphagia of db^{3J}/db^{3J} mice. The NSE-Rb transgene significantly ameliorated the obesity of db^{3J}/db^{3J} mice, as evidenced by somatic growth curves (Fig. 3*A* and *B*) and adipose depot weights (Table 1). Adult (24–27 weeks old) NSE-Rb db^{3J}/db^{3J} mice weighed 35–40% less than db^{3J}/db^{3J} mice but were significantly heavier than db^{3J}/+ mice. The weight difference between adult NSE-Rb db^{3J}/db^{3J} and db^{3J}/+ mice was more pronounced in females: female NSE-Rb db^{3J}/db^{3J} mice were ~40% heavier than female db^{3J}/+ mice, whereas male NSE-Rb db^{3J}/db^{3J} mice were only 20% heavier than male db^{3J}/+ mice (Table 1). Similar genotype and sex differences in BMI were also observed (Table 1). The absolute fat mass in female mice was significantly different among genotypes with db^{3J}/db^{3J} > NSE-Rb db^{3J}/db^{3J} > db^{3J}/+. However, fat pad mass as a percentage of body weight in female NSE-Rb db^{3J}/db^{3J} mice was not significantly different from female db^{3J}/db^{3J} mice, and fat as a percent of body weight in both of these groups was significantly higher than in db^{3J}/+ mice. In contrast, no significant difference in fat pad mass, in absolute or relative terms, was observed between male NSE-Rb db^{3J}/db^{3J} mice and lean db^{3J}/+ mice, and both of these groups had significantly less depot fat than male db^{3J}/db^{3J} mice.

Serum leptin concentrations in 24- to 27-week-old mice were highest in db^{3J}/db^{3J} mice, consistent with prior observations in *Lepr*-deficient animals (39) (Table 1). The differences in serum leptin between NSE-Rb db^{3J}/db^{3J} and db^{3J}/+ mice were proportional to the differences in adipose depot weights, and the leptin/fat mass ratio was similar between these two genotypes. The leptin/fat mass ratio of male and female db^{3J}/db^{3J} mice was significantly higher than that in lean mice of the same sex. In females, the leptin/fat mass ratio of db^{3J}/db^{3J} mice was significantly higher than that of NSE-Rb db^{3J}/db^{3J} mice. However, this difference was not seen in male animals (Table 1). Twenty-four hours of fasting decreased plasma leptin in lean (db^{3J}/+, +/+) mice, although it failed to do so in both db^{3J}/db^{3J} and NSE-Rb db^{3J}/db^{3J} mice (Table 2). Fasting for an additional 24 h did not decrease serum leptin concentrations of either db^{3J}/db^{3J} or NSE-Rb db^{3J}/db^{3J} mice

TABLE 1
Indices of adiposity in male and female mice indicating effects of LEPR deficiency and the NSE-Rb transgene

Genotype (n)	Body weight (g)	BMI	Fat pad mass		Leptin (ng/ml)	Leptin/fat mass
			g	% Body weight		
Females						
db^{3J}/+ (11)	31.1 ± 1.5	0.36 ± 0.01	4.7 ± 0.8	14.5 ± 1.8	34 ± 10	5.7 ± 1.1
db^{3J}/db^{3J} (13)	73.1 ± 2.6*	0.74 ± 0.02*	22.9 ± 1.3*	31.1 ± 0.9*	248 ± 10*	11.2 ± 0.7*
NSE-Rb db^{3J}/db^{3J} (5)	44.0 ± 1.4*†	0.47 ± 0.01*†	12.0 ± 1.0*†	27.0 ± 1.5*	90 ± 11*†	6.3 ± 1.7†
Males						
db^{3J}/+ (12)	40.2 ± 1.4	0.41 ± 0.01	5.5 ± 0.3	13.6 ± 0.6	54 ± 6	9.6 ± 0.8
db^{3J}/db^{3J} (8)	74.1 ± 1.4*	0.70 ± 0.01*	18.8 ± 0.91*	25.3 ± 1.1*	222 ± 11*	12.1 ± 1.0*
NSE-Rb db^{3J}/db^{3J} (7)	48.4 ± 1.9*†	0.47 ± 0.02*†	6.9 ± 0.6†	14.2 ± 0.8†	77 ± 9†	11 ± 0.6

Data are means ± SE. Mice were 24–27 weeks old. *P < 0.05 vs. lean mice (db^{3J}/+) of the same sex; †P < 0.05 vs. obese mice (db^{3J}/db^{3J}) of the same sex.

TABLE 2
Response to a fast in two circulating hormones, leptin and insulin

	db^{3J}/+, +/+	db^{3J}/db^{3J}	NSE-Rb db^{3J}/db^{3J}
Males			
n	4	5	4
Body weight (g)	33.9 ± 1.5	57 ± 1.6*	40.7 ± 1.7*†
Leptin			
Fed (ng/ml)	21.4 ± 3.5	111.0 ± 15.9*	44.1 ± 8.9*†
24-h Fasted (ng/ml)	10.8 ± 3.3‡	135.0 ± 19.9	43.6 ± 8.6
Glucose homeostasis			
Glucose			
Fed (mg/dl)	142 ± 3	378 ± 38*	131 ± 8
24-h Fasted (mg/dl)	93 ± 4‡	97 ± 9‡	94 ± 6‡
Insulin			
Fed (ng/ml)	2.9 ± 0.9	15.6 ± 3.8*	5.2 ± 1.8†
24-h Fasted (ng/ml)	0.2 ± 0.1‡	3.4 ± 0.4	1.1 ± 0.1
Females			
n	3	3	3
Body weight (g)	25.6 ± 0.2	62.2 ± 0.7*	36.5 ± 0.8*†
Leptin			
Fed (ng/ml)	17.2 ± 2.0	169.0 ± 10.2*	52.4 ± 3.6*†
24-h Fasted (ng/ml)	13.1 ± 2.6‡	218.0 ± 30.2*	52.4 ± 3.9*†
Glucose homeostasis			
Glucose			
Fed (mg/dl)	118 ± 5	314 ± 16	109 ± 5
24-h Fasted (mg/dl)	56 ± 4‡	83 ± 16‡	58 ± 5‡
Insulin			
Fed (ng/ml)	0.6 ± 0.1	19.1 ± 0.3*	2.3 ± 0.4*†
24-h Fasted (ng/ml)	0.3 ± 0.1	10.1 ± 3.9	2.3 ± 1.2

Data are means ± SE. Mice were 15–16 weeks old. *P < 0.05 vs. lean (db^{3J}/+, +/+) mice; †P < 0.05 vs. db^{3J}/db^{3J} mice; ‡P < 0.05 vs. fed mice of same genotype.

(data not shown). Thus, the transgene failed to restore the deficit in fasting-mediated regulation of plasma leptin seen in db^{3J}/db^{3J} mice.

The lower body weight and total fat pad mass of NSE-Rb db^{3J}/db^{3J} mice was associated with lower daily food intake (Table 3). The 24-h food intake of 11- to 12-week-old NSE-Rb db^{3J}/db^{3J} mice was not significantly different from the intake of db^{3J}/+ mice, although there was a trend toward a higher intake in male NSE-Rb db^{3J}/db^{3J} mice (P = 0.08). No significant differences among groups were observed when food intake was expressed as a power function of body weight (body weight$^{0.75}$), which is proportional to metabolic mass.

The NSE-Rb transgene corrects the cold intolerance of db^{3J}/db^{3J} mice. Mice that lack leptin or LEPR are hypothermic and have a reduced thermogenic response to cold challenge, due primarily to decreased sympathetic stimulation of brown adipose tissue (40). When 16- to 20-week-old male db^{3J}/db^{3J} mice were placed at 4°C, there was a significant 7.4 ± 1.7°C decrease in their core temperature by 1 h as measured with a rectal probe (Fig. 4*A*). The basal core temperatures of wild-type (+/+), db^{3J}/db^{3J}, and NSE-Rb db^{3J}/db^{3J} male mice were not significantly different. Both the +/+ and NSE-Rb db^{3J}/db^{3J} male mice maintained their core temperature during 2 h at 4°C, with no significant difference from the basal core temperature in either genotype at any time point.

The correction of the thermogenic defect by the transgene is attenuated in females (Fig. 4*B*). The db^{3J}/db^{3J} females have a slightly lower core temperature than the db^{3J}/+ females. There is no difference in core temperatures between db^{3J}/+ and NSE-Rb db^{3J}/db^{3J} females. When exposed to cold, the db^{3J}/db^{3J}

TABLE 3
Effects of the db^{3J} mutation and the NSE-Rb transgene on food intake

Food intake	db^{3J}/+	db^{3J}/db^{3J}	NSE-Rb db^{3J}/db^{3J}
Males			
n	6	3	5
g/day	4.1 ± 0.16	6.5 ± 0.2*	5.0 ± 0.37†
g · day^{-1} · g body wt$^{-0.75}$	0.034 ± 0.003	0.035 ± 0.001	0.032 ± 0.002
Females			
n	4	7	4
g/day	3.9 ± 0.3	6.4 ± 0.6*	4.3 ± 0.3†
g · day^{-1} · g body wt$^{-0.75}$	0.038 ± 0.003	0.034 ± 0.003	0.034 ± 0.003

Data are means ± SE. Mice were 11–12 weeks old. *P < 0.05 vs. lean (db^{3J}/+) mice; †P < 0.05 vs. db^{3J}/db^{3J} mice.

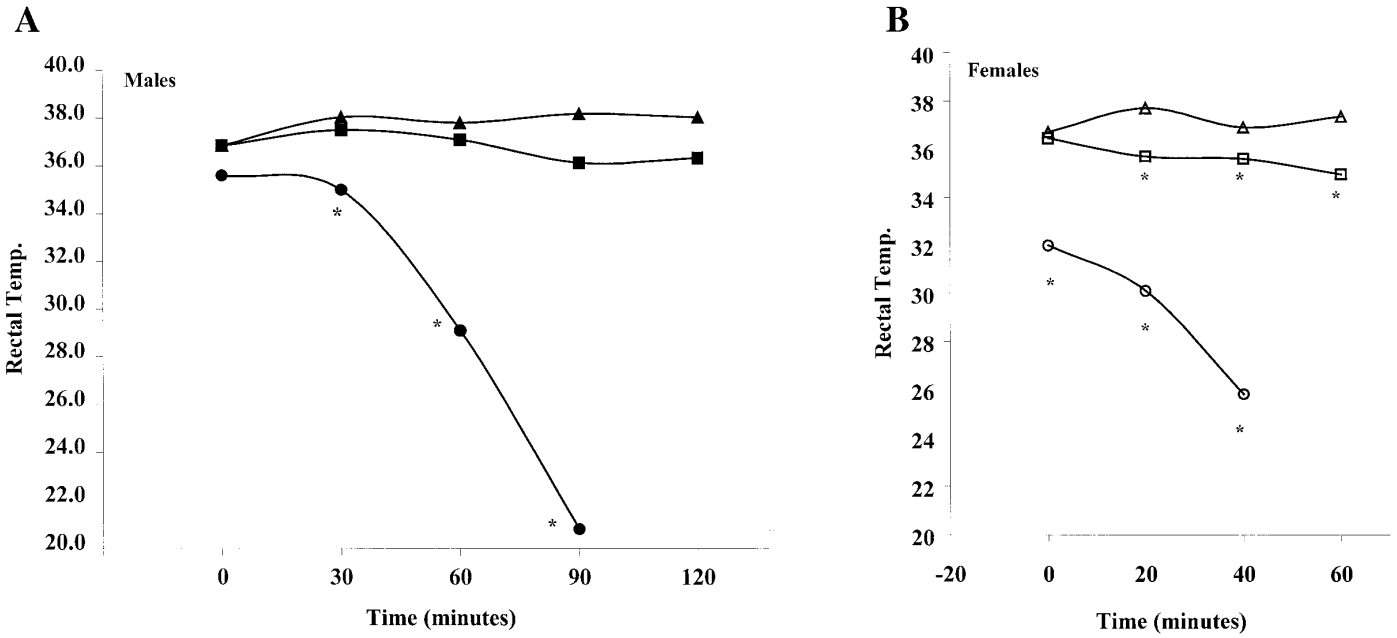

FIG. 4. Cold tolerance test in 16- to 20-week-old male (*A*) and female (*B*) +/+ (n = 4, ▲, △), db^{3J}/db^{3J} (n = 3, ●, ○), and NSE-Rb db^{3J}/db^{3J} (n = 4, ■, □) mice. *Significantly different from t = 0 of same genotype (P < 0.05).

females have a dramatic decrease in core temperature (6.2° over 40 min), whereas db^{3J}/+ females show no alterations in core temperatures. The NSE-Rb db^{3J}/db^{3J} females show a small but significant drop in core temperature (1.5° over 90 min), which is significantly different from db^{3J}/+ females.

The NSE-Rb transgene restores fertility in male db^{3J}/db^{3J} mice. Both *Lep*- and *Lepr*-deficient mice are infertile (1). To evaluate the effect of the NSE-Rb transgene on fertility in *Lepr*-null mice, we mated NSE-Rb db^{3J}/db^{3J} mice (four males and three females) to lean (db^{3J}/+) mice. All of the NSE-Rb db^{3J}/db^{3J} males sired at least two litters of normal size. However, none of the NSE-Rb db^{3J}/db^{3J} females produced litters. It was not determined if the females were cycling or were impregnated but failed to bring any fetuses to term.

The NSE-Rb transgene partially corrects the glucose intolerance of db^{3J}/db^{3J} mice. Another hallmark of *Lep*- and *Lepr*-deficient mice is insulin resistance and a strain-dependent glucose intolerance (1). The NSE-Rb transgene fully corrected the postprandial hyperglycemia of both male and female db^{3J}/db^{3J} mice (Table 2). There was, however, only a partial correction of the hyperinsulinemia. It is possible that the hyperinsulinemia in NSE-Rb db^{3J}/db^{3J} mice is due to their persistent excess adiposity. The glucose tolerance curves of male NSE-Rb db^{3J}/db^{3J} and lean mice were similar (Fig. 5*A*), although there was a trend for the NSE-Rb db^{3J}/db^{3J} mice to have higher glucose values at 40 min after glucose administration. The glucose tolerance curves of female NSE-Rb db^{3J}/db^{3J} mice were intermediate between those of db^{3J}/db^{3J} and db^{3J}/+ or +/+ mice, indicating a persistent but attenuated glucose intolerance (Fig. 5*B*).

Effects of NSE-Rb on expression of hypothalamic POMC, NPY, AGRP, and MCH. To specify the neuronal substrates that mediate the various effects of the NSE-Rb transgene, we performed quantitative analysis (by semi-quantitative RT-PCR and in situ hybridization) of the hypothalamic expression of four neuropeptide genes (Fig. 6) that are regulated by leptin and abnormally expressed in LEPR-deficient rodents: POMC, AGRP, NPY, and MCH. POMC transcripts (Fig. 6*A*) are normalized (increased twofold over db^{3J}/db^{3J} male mice) by the NSE-Rb transgene in male db^{3J}/db^{3J} transgenics, whereas female db^{3J}/db^{3J} transgenics had a large increase (fivefold) in POMC mRNA concentrations over lean and db^{3J}/db^{3J} females. Although we did not observe any differences in POMC mRNA between the db^{3J}/+ and db^{3J}/db^{3J} genotypes, the observation of a significant increase of POMC mRNA in the transgenic females indicates the bestowal of leptin sensitivity of POMC neurons by the NSE-Rb transgene in both male and female db^{3J}/db^{3J} mice. Leptin-receptor deficiency increased *Agrp* expression (Fig. 6*C*), whereas AGRP transcripts were reduced by the transgene but not normalized to the amounts observed in db^{3J}/+ animals in both male and female NSE-Rb db^{3J}/db^{3J} mice. We also found a reduction, but not full normalization, of NPY mRNA (Fig. 6*B*) in male db^{3J}/db^{3J} mice due to the transgene by in situ hybridization, although we were unable to observe a transgene effect by RT-PCR analysis (data not shown). Contrary to the NSE-Rb transgene's effects on arcuate nucleus neurons, there was no difference in MCH mRNA (Fig. 6*D*) concentrations between db^{3J}/db^{3J} and NSE-Rb db^{3J}/db^{3J} mice of both sexes, although lean db^{3J}/+ mice of both sexes had significantly lower MCH mRNA concentrations than the db^{3J} mutant mice (transgenic and nontransgenic). Therefore, the partial corrections in the obesity/diabetes phenotype that are observed in the NSE-Rb db^{3J}/db^{3J} mouse may be due to the restoration of leptin sensitivity to arcuate nucleus neurons expressing POMC/cocaine- and amphetamine-regulated transcript (CART) and AGRP/NPY, whereas the residual deficiencies could be due to dysregulation of MCH neurons in the lateral hypothalamus and some AGRP/NPY neurons in the arcuate nucleus.

Presence of other LEPR isoforms on effects of the NSE-Rb transgene. It is possible that the failure of the NSE-

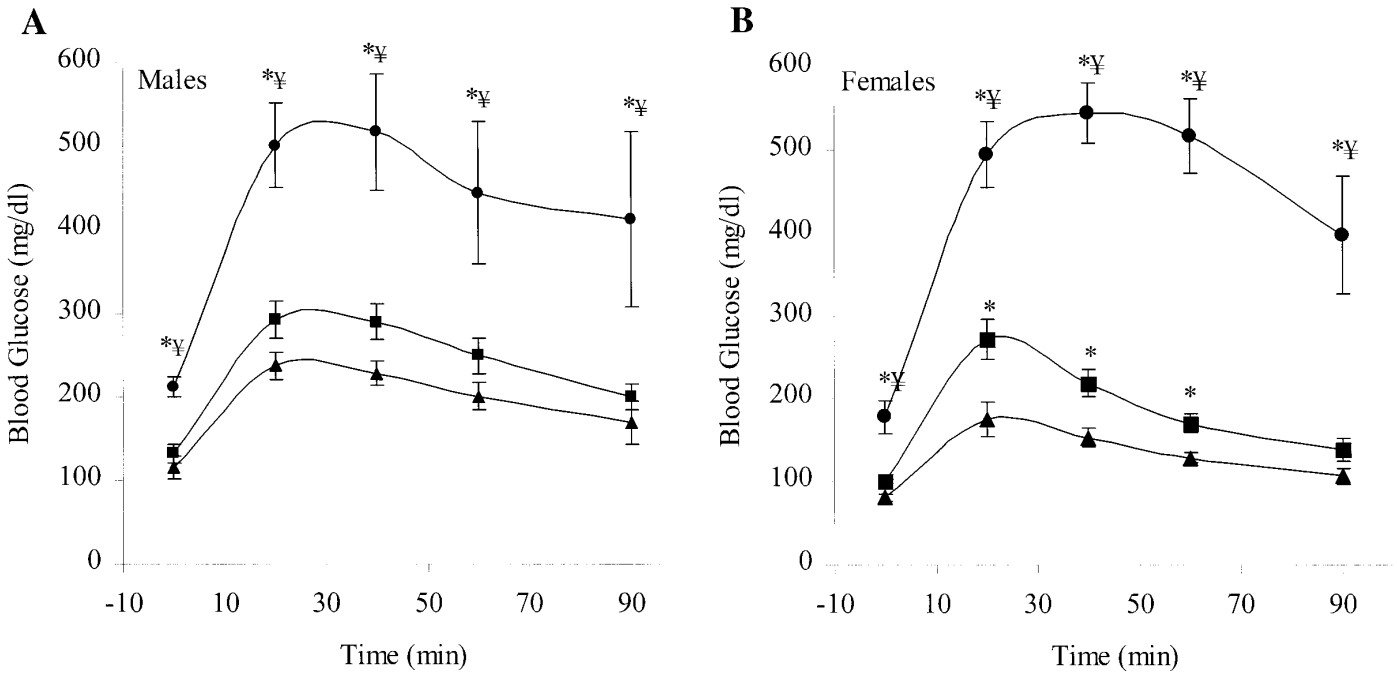

FIG. 5. ***A*: Intraperitoneal glucose tolerance test in 13- to 22-week-old male lean (db^{3J}/+, +/+) (n = 4, ▲), db^{3J}/db^{3J} (n = 3, ●), and NSE-Rb db^{3J}/db^{3J} (n = 5, ■) mice. *B*: Intraperitoneal glucose tolerance test in 18-week-old female lean (db^{3J}/+, +/+) (n = 3, △), db^{3J}/db^{3J} (n = 6, ○), and NSE-Rb db^{3J}/db^{3J} (n = 4, □) mice. *Significantly different from lean mice.**

Rb transgene to correct completely the obesity in LEPR-null (db^{3J}/db^{3J}) mice is due to the absence of other LEPR isoforms (e.g., LEPR-Ra, Rc, Rd, or Re). To explore this possibility, we evaluated the effect of the NSE-Rb transgene on the obesity of *db/db* mice that are deficient in only the B isoform of LEPR. Similar to observations in db^{3J}/db^{3J} mice, the presence of the NSE-Rb transgene did not fully correct the obesity phenotype of 16- to 18-week-old male *db/db* mice. The body weights of *db/db* (n = 5), NSE-Rb *db/db* (n = 4), and *db/+* or +/+ (n = 3) mice were 70 ± 2, 53 ± 1, and 43 ± 2 g, respectively, and the adipose depot masses were 13 ± 0.5, 6.7 ± 0.6, and 5.8 ± 0.1 g, respectively. Because NSE-Rb *db/db* mice possess the full complement of short LEPR isoforms, the lack of a full correction of obesity in NSE-Rb db^{3J}/db^{3J} mice cannot be explained by the absence of these short LEPR isoforms. The possibility that the short isoforms may subtly modify the phenotype of NSE-Rb *db/db* animals relative to NSE-Rb db^{3J}/db^{3J} animals cannot, however, be excluded.

DISCUSSION

Two critical questions regarding the function of the leptin-LEPR axis are: *1*) Are all of the major physiological functions of the axis conveyed through the central nervous system (CNS)? *2*) Are isoforms other than Rb required for mediating the relevant signals wherever conveyed? Mice and rats with spontaneously occurring *Lepr* mutations affecting the expression of all (db^{3J}, db^{Pas}, fa^{f}, *fa*), or only one (*db*), of the LEPR isoforms have provided useful models for determining the physiological function(s) of LEPR isoforms in various tissues. In such animals, expression of a transgene encoding a LEPR splice variant can be restricted to a desired organ(s) by using an appropriate enhancer/promoter element in animals otherwise null for LEPR, thus revealing the role(s) of LEPR isoforms in individual tissues in vivo. We have used this strategy to demonstrate that animals possessing a LEPR-B transgene that is expressed predominantly in the brain of LEPR-null animals (db^{3J}/db^{3J}) has a significant effect on body weight, glucose homeostasis, thermoregulatory thermogenesis, and reproduction, and that coexpression of the short isoforms of LEPR (NSE-Rb *db/db* mice) does not appear to modify this response.

The control of feeding behavior and energy expenditure by circulating leptin appears to be mediated primarily by LEPR-B signaling within the hypothalamus. The demonstrations that LEPR-B expression is highest in the hypothalamus (19), that peripheral and central leptin injections produce similar effects on feeding and energy expenditure in *Lep*-null mice (7–9), that *db/db* mice and *Lepr*-null mice have an apparently identical obesity/diabetes phenotype (1), and that peripheral leptin injection activates STAT3 in hypothalamic neurons but not in peripheral tissue (41) are consistent with this view. Within the hypothalamus, leptin seems to act by modulating the expression, release, and actions of several orexigenic and anorexigenic neuropeptides (42).

The NSE-Rb db^{3J}/db^{3J} transgenic mice express the LEPR-B mRNA at high levels throughout the brain and at much lower levels in adrenals, testis, and white adipose tissue. The NSE-Rb *db/db* transgenic mice have the same pattern of LEPR-B expression but possess the full complement of short LEPR isoforms in all tissues. The demonstration that the body weights of the NSE-Rb db^{3J}/db^{3J} and NSE-Rb *db/db* mice are similar suggests that the shorter LEPR isoforms are of limited importance in body weight regulation. The failure to achieve a full correction of the obesity phenotype of *Lepr*-null mice may be attributed to one or more of the following potential factors: *1*) absence of the LEPR-B transgene in critical hypothalamic neurons due to mosaic expression; *2*) failure of the transgene to enable production of the

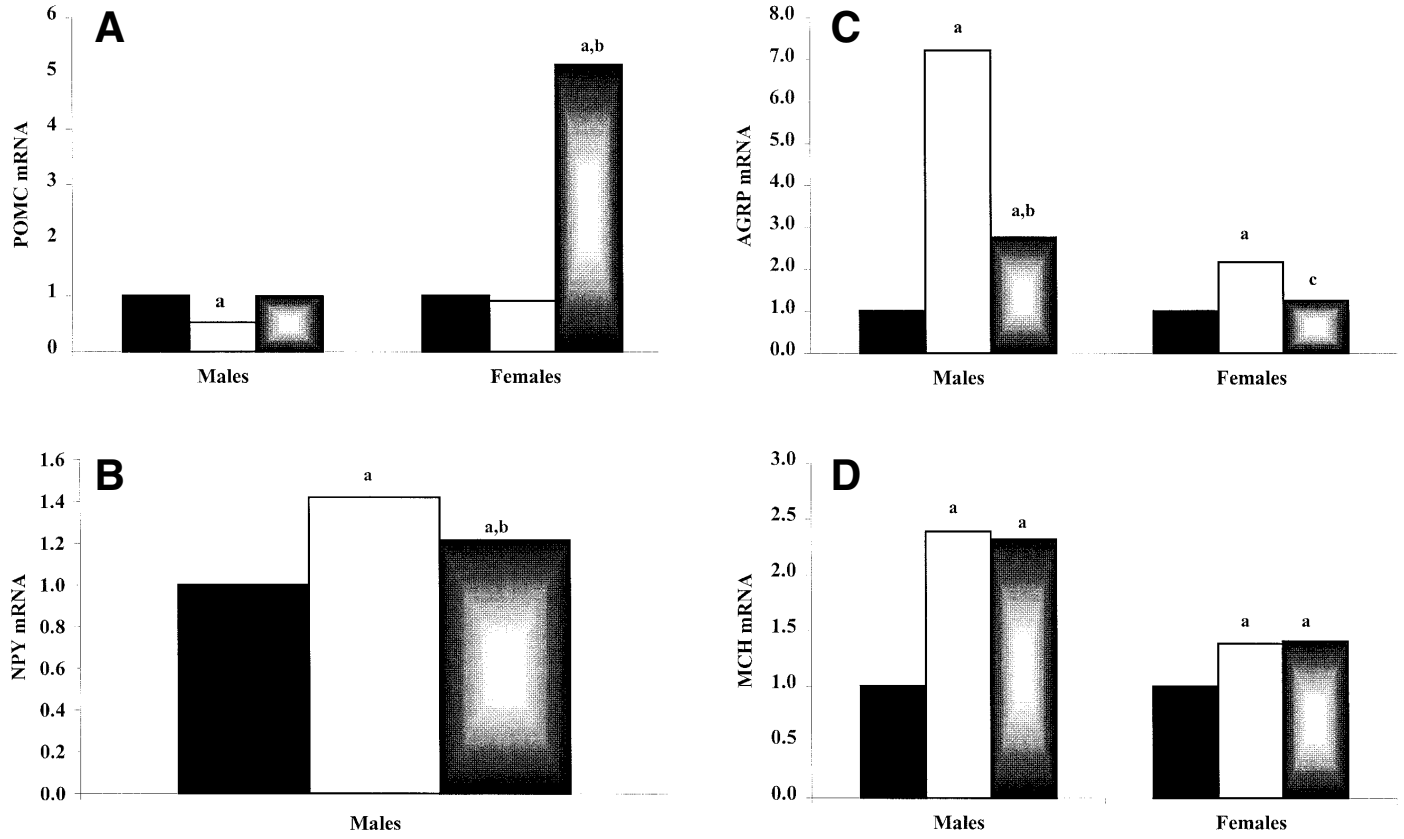

FIG. 6. Effects of NSE-Rb transgene on hypothalamic neuropeptide gene expression. Columns with symbols have the following designations: a, different from db^{3J}/+ ($P < 0.05$); b, different from db^{3J}/db^{3J} ($P < 0.05$); and c, not different from db^{3J}/+ and db^{3J}/db^{3J}. Genotypes are designated as follows: ■, db^{3J}/+; □, db^{3J}/db^{3J}; ▨, NSE-Rb db^{3J}/db^{3J}. The medians for four or five animals in each group are represented. *A*: POMC RNA quantified by RT-PCR. *B*: NPY RNA quantified by *in situ* hybridization, males only. *C*: AGRP RNA quantified by RT-PCR. *D*: MCH RNA quantified by RT-PCR.

appropriate number of leptin receptors in relevant neurons; and *3*) absence of LEPR-B transgene expression in critical peripheral tissue(s).

Both CNS and direct peripheral actions of leptin have been proposed to mediate the hormone's effects on glucose homeostasis. Because the NSE-Rb transgene is not expressed in the pancreas or in skeletal muscle, the effects of circulating leptin on glucose metabolism in NSE-Rb db^{3J}/db^{3J} mice must be mediated by LEPR-B–mediated signaling events in the CNS or white adipose tissue. Although the endogenous NSE gene (*Eno2*) is expressed in all peripheral and central neurons, the DNA fragment used in constructing the NSE-Rb transgene does not fully replicate the pattern of expression of the natural *Eno2* gene. Moreover, integration site effects have important consequences for transgene expression, emphasizing the necessity of characterizing each transgene integration site independently. The increased skeletal muscle glucose uptake in response to intracerebroventricular leptin administration suggests that leptin's effects on skeletal muscle are mediated primarily through the CNS (43). Although direct actions of leptin on adipocytes are possible, it is likely that circulating leptin affects the adipose tissue glucose metabolism of NSE-Rb db^{3J}/db^{3J} mice via CNS-related mechanisms, because the transgene reduces the ratio of circulating leptin to fat pad mass (Table 1) in NSE-Rb db^{3J}/db^{3J} mice. Reports demonstrating no direct effects of leptin on glucose transport and metabolism in adipocytes (44–46), or modest effects on glucose metabolism using supraphysiological concentrations of leptin (47), are consistent with this model.

NSE-Rb db^{3J}/db^{3J} mice lack LEPR-B expression, and thus LEPR-mediated signaling, in brown adipose tissue. The complete restoration of cold tolerance in NSE-Rb db^{3J}/db^{3J} male mice is consistent with restoration of LEPR-mediated efferent sympathetic nervous traffic in the LEPR-deficient rodents (48,49). Furthermore, because no LEPR isoforms are expressed in brown adipose tissue of NSE-Rb db^{3J}/db^{3J} mice (Fig. 2), the restoration of cold tolerance in these mice indicates that reported direct effects of leptin on glucose and lipid metabolism in brown adipose tissue in vitro (47) can be replaced by sympathetic activation mediated by leptin-responsive neurons. Similarly, sympathetic activation with a sympathetic agonist or a cold environment can circumvent the thermogenic defect of leptin deficiency (50,51).

The NSE-Rb transgene restored fertility in male db^{3J}/db^{3J} mice. It is possible that the restoration of fertility in male NSE-Rb db^{3J}/db^{3J} mice is due to expression of the NSE-Rb transgene in testis (Fig. 2). Recent work, however, demonstrates that plasma leptin enters the testes in a nonsaturable manner and that LEPR is not expressed in Leydig or Sertoli cells (52), which raises questions regarding the relevance of LEPR as a transporter or as a signaling mechanism in this tissue and suggests that the effect of the NSE-Rb transgene on fertility in male LEPR-null mice is due to the restoration of

central LEPR-B signaling. The failure of the transgene to restore fertility in female LEPR-null mice may be due to the persistent adiposity seen in these mice or to the failure to correct completely the defects in the hypothalamic-pituitary-gonadal axis, and not to a requirement for LEPR-B signaling in ovarian tissue. These inferences are supported by the facts that ovaries transplanted from *db/db* mice to +/+ mice function normally (53) and that mice made obese by high-fat feeding fail to cycle normally (54). Additionally, preliminary data show that NSE-Rb *db/db* female mice are not fertile whereas NSE-Rb *db/db* males are, indicating that the lack of short LEPR isoforms in the ovaries of NSE-Rb db^{3J}/db^{3J} mice is not responsible for the failure to restore fertility. The sex differences that the NSE-Rb transgene produces are intriguing. Although we have shown that the transgene did not integrate into the X chromosome (male hemizygotes produce male and female transgenic progeny), it is possible that the transgene integrated at a site that confers sex-specific modulation of transgene expression. There were no data that indicated that the NSE promoter has sex-specific effects on transgene expression.

The quantitative analysis of neuropeptide gene expression in the hypothalamus suggests a critical role for POMC and AGRP/NPY (55) arcuate nucleus neurons in the obesity/diabetes syndrome of leptin-receptor deficiency (42). Although these are the neuropeptide genes whose transcript concentrations were measured, it is likely that the entire set of neurotransmitter activities of these two neuronal populations is affected by the NSE-Rb transgene, such as CART in POMC neurons (56–58), because leptin hyperpolarizes neurons (59), thereby inhibiting neuronal activity and synaptic transmission. It is remarkable that several aspects of LEPR deficiency, such as glucose tolerance and fertility in males, can be corrected by near-normalization of leptin sensitivity in POMC and AGRP/NPY neurons. It should be noted that NPY deficiency does not correct glucose intolerance in leptin-deficient mice (60), whereas overexpression of Agouti (61) and AGRP (34) does not produce overt diabetes, suggesting that the activity of hypothalamic POMC neurons is important to the maintenance of normoglycemia. In support of this supposition is the observation that mice lacking the melanocortin receptor 4 (MC4R)—the receptor for α-melanocyte stimulating hormone (a peptide processed from the POMC preprotein) and AGRP—develop hyperglycemia and are also infertile (62). However, an intact hypothalamus is necessary for the development of hyperglycemia in LEPR-deficient rodents (in diabetes-susceptible strains) because ventromedial hypothalamic lesions, either chemically or electrolytically induced, prevent the development of diabetes without affecting the degree of obesity (63). Ventromedial hypothalamic lesions also ablate the arcuate nucleus, removing both POMC and AGRP/NPY neuronal populations. Because we have not controlled for adiposity between transgenic and nontransgenic LEPR-deficient mice in our study, it remains a formal possibility that the diminished adiposity in NSE-Rb mice may be sufficient to account for improved glycemic control. Based on a comparative survey of the current literature cited previously, we believe that it is likely that the combined actions of at least two neuronal populations in the arcuate nucleus, including POMC neurons and AGRP/NPY neurons, are important for regulating glucose homeostasis.

The persistent adiposity, insulin resistance, and female infertility of NSE-Rb LEPR-deficient mice may be ascribed to the partial (for AGRP/NPY neurons) or complete (for MCH neurons) leptin insensitivity of a subset of hypothalamic neurons. The lack of fasting-induced suppression of circulating leptin by the NSE-Rb transgene suggests that LEPR may be a potential regulator of leptin secretion. Although our data are compatible with the possibility of peripheral actions of leptin/leptin receptor contributing to the obesity/diabetes syndrome, a direct test of this hypothesis is required by evaluation of the phenotype of mice expressing LEPR-B in adipocytes only. In addition, we are performing further studies where higher levels of LEPR-B transgene expression, i.e., in the homozygous state, may affect adiposity in lean and genetically obese mice.

In summary, we have demonstrated that expression of LEPR-B predominantly in the brain of mice otherwise null for all LEPR isoforms affects all fundamental aspects of the obesity/diabetes syndrome, as the NSE-Rb transgene partially rescues the obesity/diabetes phenotype, restores fertility in male animals, and fully corrects the impaired thermoregulatory thermogenesis in males. Future experiments in transgenic mice in which expression of LEPR-B is restricted to specific hypothalamic neurons (e.g., POMC- or AGRP/NPY-expressing neurons) or peripheral tissues (e.g., adipose tissue) will help to define the minimum molecular and neuronal phenotypes required for mediation of leptin's behavioral and metabolic effects.

ACKNOWLEDGMENTS

This work was funded by the National Institutes of Health Grants NS10675 (T.J.K.), DK52431 (R.L. and S.C.), and DK26687 (R.L. and S.C.), and an American Diabetes Association mentored traineeship (R.L.).

The authors would like to acknowledge the contributions of Norichika Okada and James Koutras in the construction of the NSE-Rb transgene; Joel Elmquist and Charlotte Lee for in situ hybridization analysis of the NSE-Rb transgene; and Eric Corp for leptin binding visualization.

REFERENCES

1. Leibel RL, Chung WK, Chua SC Jr: The molecular genetics of rodent single gene obesities. *J Biol Chem* 272:31937–31940, 1997
2. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994
3. Rosenbaum M, Nicolson M, Hirsch J, Murphy E, Chu F, Leibel RL: Effects of weight change on plasma leptin concentrations and energy expenditure. *J Clin Endocrinol Metab* 82:3647–3654, 1997
4. Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J: Transient increase in obese gene expression after food intake or insulin administration. *Nature* 377:527–529, 1995
5. Rosenbaum M, Leibel RL: The role of leptin in human physiology. *N Engl J Med* 341:913–915, 1999
6. Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS: Role of leptin in the neuroendocrine response to fasting. *Nature* 382:250–252, 1996
7. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F: Effects of the obese gene product on body weight regulation in *ob/ob* mice. *Science* 269:540–543, 1995
8. Chehab FF, Lim ME, Lu R: Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 12:318–320, 1996
9. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P: Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546–549, 1995
10. Emilsson V, Liu YL, Cawthorne MA, Morton NM, Davenport M: Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory

action of leptin on insulin secretion. *Diabetes* 46:313–316, 1997
11. Muoio DM, Dohm GL, Tapscott EB, Coleman RA: Leptin opposes insulin's effects on fatty acid partitioning in muscles isolated from obese *ob/ob* mice. *Am J Physiol* 276:E913–E921, 1999
12. Cohen B, Novick D, Rubinstein M: Modulation of insulin activities by leptin. *Science* 274:1185–1188, 1996
13. Muller G, Ertl J, Gerl M, Preibisch G: Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *J Biol Chem* 272:10585–10593, 1997
14. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, et al.: Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83:1263–1271, 1995
15. Ihle JN: Cytokine receptor signalling. *Nature* 377:591–594, 1995
16. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632–635, 1996
17. Lollmann B, Gruninger S, Stricker-Krongrad A, Chiesi M: Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and e in different mouse tissues. *Biochem Biophys Res Commun* 238:648–652, 1997
18. Chua SC Jr, Koutras IK, Han L, Liu SM, Kay J, Young SJ, Chung WK, Leibel RL: Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts. *Genomics* 45:264–270, 1997
19. Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC: Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci U S A* 93:6231–6235, 1996
20. White DW, Wang DW, Chua SC Jr, Morgenstern JP, Leibel RL, Baumann H, Tartaglia LA: Constitutive and impaired signaling of leptin receptors containing the Gln→Pro extracellular domain fatty mutation. *Proc Natl Acad Sci U S A* 94:10657–10662, 1997
21. Leiter EH, Coleman DL, Eisenstein AB, Strack I: A new mutation (db3J) at the diabetes locus in strain 129/J mice. I. Physiological and histological characterization. *Diabetologia* 19:58–65, 1980
22. Aubert R, Herzog J, Camus MC, Guenet JL, Lemonnier D: Description of a new model of genetic obesity: the dbPas mouse. *J Nutr* 115:327–333, 1985
23. Brown JA, Chua SC Jr, Liu SM, Andrews MT, Vandenbergh JG: Spontaneous mutation in the db gene results in obesity and diabetes in CD-1 outbred mice. *Am J Physiol Regul Integr Comp Physiol* 278:R320–R330, 2000
24. Zucker LM, Zucker TM: Fatty, a new mutation in the rat. *J Hered* 52:272–278, 1961
25. Koletsky S: Obese spontaneously hypertensive rats: a model for study of atherosclerosis. *Exp Mol Pathol* 19:53–60, 1973
26. Murakami T, Yamashita T, Iida M, Kuwajima M, Shima K: A short form of leptin receptor performs signal transduction. *Biochem Biophys Res Commun* 231:26–29, 1997
27. Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, Sutcliffe JG: Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron* 5:187–197, 1990
28. Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD: Introns increase transcriptional efficiency in transgenic mice. *Proc Natl Acad Sci U S A* 85:836–840, 1988
29. Liu K, Sandgren EP, Palmiter RD, Stein A: Rat growth hormone gene introns stimulate nucleosome alignment in vitro and in transgenic mice. *Proc Natl Acad Sci U S A* 92:7724–7728, 1995
30. Hogan B, Beddington R, Costantini F, Lacy E: *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Press, 1994
31. Schreyer SA, Chua SC Jr, LeBoeuf RC: Obesity and diabetes in TNF-alpha receptor-deficient mice. *J Clin Invest* 102:402–411, 1998
32. Chua SC Jr, Brown AW, Kim J, Hennessey KL, Leibel RL, Hirsch J: Food deprivation and hypothalamic neuropeptide gene expression: effects of strain background and the diabetes mutation. *Brain Res Mol Brain Res* 11:291–299, 1991
33. Alonso S, Minty A, Bourlet Y, Buckingham M: Comparison of three actin-coding sequences in the mouse: evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* 23:11–22, 1986
34. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS: Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278:135–138, 1997
35. Notake M, Tobimatsu T, Watanabe Y, Takahashi H, Mishina M, Numa S: Isolation and characterization of the mouse corticotropin-beta-lipotropin precursor gene and a related pseudogene. *FEBS Lett* 156:67–71, 1983
36. Raeymaekers L: Quantitative PCR: theoretical considerations with practical implications. *Anal Biochem* 214:582–585, 1993
37. Winer J, Jung CK, Shackel I, Williams PM: Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* 270:41–49, 1999
38. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 6:986–994, 1996
39. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al.: Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nat Med* 1:1155–1161, 1995
40. Holt SJ, York DA: Studies on the sympathetic efferent nerves of brown adipose tissue of lean and obese Zucker rats. *Brain Res* 481:106–112, 1989
41. Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM: Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice. *Nat Genet* 14:95–97, 1996
42. Baskin DG, Hahn TM, Schwartz MW: Leptin sensitive neurons in the hypothalamus. *Horm Metab Res* 31:345–350, 1999
43. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374–377, 1997
44. Mick G, Vanderbloomer T, Fu CL, McCormick K: Leptin does not affect adipocyte glucose metabolism: studies in fresh and cultured adipocytes. *Metabolism* 47:1360–1365, 1998
45. Ranganathan S, Ciaraldi TP, Henry RR, Mudaliar S, Kern PA: Lack of effect of leptin on glucose transport, lipoprotein lipase, and insulin action in adipose and muscle cells. *Endocrinology* 139:2509–2513, 1998
46. Zierath JR, Frevert EU, Ryder JW, Berggren PO, Kahn BB: Evidence against a direct effect of leptin on glucose transport in skeletal muscle and adipocytes. *Diabetes* 47:1–4, 1998
47. Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Pernin A, Chin WW, Cusin I, Rohner-Jeanrenaud F, Burger AG, Zapf J, Meier CA: Direct effects of leptin on brown and white adipose tissue. *J Clin Invest* 100:2858–2864, 1997
48. Commins SP, Watson PM, Padgett MA, Dudley A, Argyropoulos G, Gettys TW: Induction of uncoupling protein expression in brown and white adipose tissue by leptin. *Endocrinology* 140:292–300, 1999
49. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI: Receptor-mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 100:270–278, 1997
50. Coleman DL: Thermogenesis in diabetes-obesity syndromes in mutant mice. *Diabetologia* 22:205–211, 1982
51. Smith CK, Romsos DR: Cold acclimation of obese (*ob/ob*) mice: effects on skeletal muscle and bone. *Metabolism* 33:858–863, 1984
52. Banks WA, McLay RN, Kastin AJ, Sarmiento U, Scully S: Passage of leptin across the blood-testis barrier. *Am J Physiol* 276:E1099–E1104, 1999
53. Bahary N, Leibel RL, Joseph L, Friedman JM: Molecular mapping of the mouse db mutation. *Proc Natl Acad Sci U S A* 87:8642–8646, 1990
54. Glick Z, Yamini S, Lupien J, Sod-Moriah U: Estrous cycle irregularities in overfed rats. *Physiol Behav* 47:307–310, 1990
55. Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T: The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proc Natl Acad Sci U S A* 95:15043–15048, 1998
56. Broberger C: Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. *Brain Res* 848:101–113, 1999
57. Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK: Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 21:1375–1385, 1998
58. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S: Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393:72–76, 1998
59. Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford ML: Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature* 390:521–525, 1997
60. Erickson JC, Hollopeter G, Palmiter RD: Attenuation of the obesity syndrome of *ob/ob* mice by the loss of neuropeptide Y. *Science* 274:1704–1707, 1996
61. Siracusa LD: The agouti gene: turned on to yellow. *Trends Genet* 10:423–428, 1994
62. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141, 1997
63. Coleman DL, Hummel KP: The effects of hypothalamic lesions in genetically diabetic mice. *Diabetologia* 6:263–267, 1970